PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Docket No.: **Q87648**
Arne HERMANSEN et al	Conf. No.: **9199**
Appln. No.: **10/533,166**	Group Art Unit: **1637**
Filed: **April 29, 2005**	Examiner: **Pande, S.**

For: **ASSAY METHOD**

DECLARATION UNDER 37 C.F.R. § 1.132

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Sonja Klemsdal, a Norwegian citizen, declare as follows:

1. I am a co-inventor for the patent application entitled "Assay method", filed on 31 October 2003 as PCT/GB2003/004712 and claiming priority from GB application Nos. 0225550.3 and 0225551.1, both filed 1 November 2002. This international application subsequently entered the United States national phase as application No. 10/533,166 (hereinafter "The Application").

2. I am familiar with the Office action dated January 10, 2008, in which the Examiner rejects Claims 7, 10, 13-14 under 35 USC § 103(a) as being unpatentable over Matsumoto *et al.* (2000) Mycol. Res. 104 (11):1333-1341 in view of Buck *et al.* (1999) Biotechniques 27: 528-536 and rejects Claims 11-12, 17 and 22 under 35 USC 103(a) as being unpatentable over Matsumoto *et al.* (2000) Mycol. Res. 104 (11):1333-1341 in view of Buck *et al.* (1999) Biotechniques 27: 528-536, further in view of Inoko *et al.* (WO 01/92572 A1 - with English equivalent document US

2003/0228585 A1). In order to demonstrate that the invention is not made obvious over the cited references, I have performed a number of experiments in relation to the subject matter claimed in The Application. The methods, results and a discussion thereof are set out below. The present experiments relate to the *P. sylvaticum* strain of the Pythium fungus. The results presented herein are expected to generally hold true for other pathogenic Pythium strains.

3. Experiment A sets out to address whether primers designed to bind across the ITS2 region of *P. sylvaticum* would demonstrate species specificity.

4. Experiment B was designed to investigate how critical the exact 5' and 3' sequences of the *P. sylvaticum* species-specific primers are in order to retain species specificity.

Materials and Methods

5. For Experiment A, the online primer picking tool "Primer3" (Steve Rozen and Helen J. Skaletsky (2000) - see http://fokker.wi.mit.edu/primer3/input.htm) was used to pick primers around the ITS regions of *P. sylvaticum* py77 (Accession No.: AB108008). The product size range was chosen to be 300-400 nucleotides. The results of the Primer3 analysis are given in Annex I.

Essentially, the program identified 5 primer pairs. The position of the optimum pair (#1) is identified with chevrons on the 907bp sequence given in Annex I. The optimum primer pair (pair #1) and the pairs identified as additional oligo pair 2 and 4 (pairs #2 and #3, respectively) were chosen for further study. These are all 21-mers and have the sequences (5' to 3'):

1_F	GAGAGTTGCAGATGTGAAGTG
1_R	TCAAACCCGGAGTACACTAAT
2_F	CCTTTTAAATGGACACGACTT
2_R	TCAAACCCGGAGTACACTAAT
3_F	TTGCAGAATTCAGTGAGTCAT
3_R	AGACACCCAATAAGCAACATT

6. Primers were ordered from Invitrogen and were used in a PCR protocol as set out below. Approximately 5 ng genomic DNA from a number of Pythium and Phytophthora species was used as a template. The isolates tested were *P. sylvaticum* (three isolates), other *Pythium* species causing cavity spot in carrots (eight isolates: *P. intermedium, P. violae, P. sulcatum* and *P.* "vipa"), other closely related *Pythium* species and species of *Pythium* and *Phytophthora* known to be frequently present in soil where carrots are grown.

7. PCR reactions for the three assays representing each of the three different primer pairs were performed in a total volume of 25 µl with final concentration 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each dNTP, 0.1 mg/ml bovine serum albumin and 1.5 mM $MgCl_2$. For each reaction 25 pmol of each primer and 0.6U AmpliTaq polymerase (Applied Biosystems) were used. Amplifications were performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) programmed for initial denaturation at 94°C for 5 min followed by 45 cycles of 20 sec 94°C, 30 sec 56°C and 30 sec at 72°C.

8. PCR products were separated and visualised using conventional gel electrophoresis techniques (1.2% agarose gels). The samples were compared with a negative control (water) and markers (lanes on the gels marked "M" contained a standard 100bp ladder).

9. For Experiment B, quantitative PCR assays were carried out as indicated below using the primers (5' to 3'):

IXb GCCAATTGCACAAGTACAAA (SEQ ID NO: 18)

4 TGCCAATTGCACAAGTACAAA

5 CTGCCAATTGCACAAGTACAAAA

6 TAGTAGTGGGCGACTCGTTGT

7 TAGTAGTGGGCGACTCGTTGTC

10. Quantitative PCR reactions (TaqMan) were performed in a total volume of 25 µl with final concentrations of primers 300 mM (Invitrogen) and of probe 100 mM (Applied Biosystems). The sequence of the TaqMan probe used was 5' 6FAM-TGGGTGCATCTGTG 3'. The mastermix used was RT-QP2x (Eurogentec). The samples were run in the real-time PCR instrument 7900HT from Applied Biosystems, with a standard 96-well block with a standard qPCR program consisting of 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The results were analysed by the SDS software (Sequence Detection System Version 2.2.2) (Applied Biosystems).

Results - Experiment A

11. The results of Experiment A are shown in Annex II (gel A shows the results from primer pair #1, gel B from pair #2 and gel C from pair #3) and collated in Table A ("-" indicates no amplification, "(-)" indicates faint amplification and "+" indicates positive amplification of fragments of the expected size):

Table A:

Lane	Template	1 F/R	2 F/R	3 F/R
1	*P. intermedium*	+	+	+
2	*P.* "vipa"	-	-	+
3	*P.* "vipa"	+	+	+
4	*P. violae*	+	+	+
5	*P. violae*	-	-	+
6	*P. sulcatum*	-	-	-
7	*P. sylvaticum*	+	+	+
8	*P. sylvaticum*	-	-	(-)
9	*P. sylvaticum*	+	+	+
10	*Pythium* spp.	(-)	-	+
11	*P. dissotocum*	+	(-)	+
12	*Pythium* spp.	+	-	+
13	*P. aphanidermatum*	+	+	+
14	*P. dissotocum*	+	+	+
15	*P. middletonii*	-	-	+
16	*Phytophthora megasperma*	+	-	-
17	*Pythium* spp.	+	-	+
18	*P. aquatile*	-	-	-
19	*Pythium* spp.	-	+	+
20	*P. mamillatum*	-	+	+
21	*Pythium gruppe* T	+	+	+
22	*Pythium* spp.	(-)	-	-
23	*Phytophthora cryptogea*	-	-	(-)
24	*Pythium* spp.	-	+	+
25	*P. sylvaticum*	+	-	+
26	*P. intermedium*	(-)	-	+
27	*P. violae*	-	-	-
28	Negative control (water)	-	-	-

Results - Experiment B

12. The results of the quantitative PCR are shown in Table B ("poor" indicates a lesser degree of species specificity when compared with the primers of the invention):

Table B:

Primer	Species-specific amplification
IXb	yes
4	no
5	no
6	poor
7	poor

Discussion

13. The Primer3 analysis of *P. sylvaticum* DNA sequence AB108008 suggested 5 PCR primer pairs, three of which pairs were chosen (primers 1_F to 3_R). These primer pairs were tested by PCR with a collection of Pythium species as well as two Phytophthora species.

14. As shown in Table A above, all three pairs of primers amplified DNA from more than one species of Pythium, even amplifying DNA from the Phytophthora samples. It can also be seen that not all *P. sylvaticum* strains tested could have DNA amplified, i.e. could be detected, by these primer pairs (see for example the *P. sylvaticum* strain in lane 25).

15. The results of Experiment A demonstrate conclusively that primers generated by a standard primer-generating program in the ITS1-5.8S rRNA-ITS2 DNA region are not generally useful for species-specific amplification of DNA from a given Pythium species (in this example, *P. sylvaticum*).

16. The results of Experiment B (Table B above) demonstrate that making very minor changes to sequence of primer IXb (i.e. single base additions to either to the 5' or to the 3' ends) results in a loss of species-specificity. This indicates that primer IXb is in the optimum position for sequence specificity. It is neither a random nor an arbitrary choice.

17. The locations of the primers within the variable regions of ITS2, as claimed in The Application, are not random, but were carefully selected to give primers that are species-specific in binding. Although regions of DNA, for example of the ITS2

region, were known which were not highly conserved between species (e.g. between *P. ultimum* and *P. sylvaticum*), but which were grossly conserved within species (e.g. between *P. sylvaticum* strains), the choice of species-specific primers as claimed would not have been obvious to the skilled person.

18. It has thus been demonstrated that random priming does not yield species-specific results. It has also been shown that the end-points (5' and 3') of a species-specific primer are important in ensuring that the primer retains its species-specificity.

19. Furthermore, primers for use in diagnostic or species-specific PCR have an extra requirement. The template in such PCR is mixed DNA from hundreds, thousands or even millions of different organisms (species). The primers described in The Application work well in the species-specific detection of the specific Pythium species in such a complex medium, such as a total DNA sample extracted from soil.

20. In conclusion, I believe that the species-specific primers as claimed in The Application are not obvious in light of what was known at the priority date of the invention.

21. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed at , on the day of July 2008

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Sonja Klemsdal

Annex I

Primer3 Output

WARNING: Numbers in input sequence were deleted.

PRIMER PICKING RESULTS FOR AB108088

No mispriming library specified
Using 1-based sequence positions

OLIGO	start	len	tm	gc%	any	3'	seq	
LEFT PRIMER	490	21	55.99	47.62	4.00	2.00	gagagttgcagatgtgaagtg	#1
RIGHT PRIMER	888	21	56.26	42.86	4.00	2.00	tcaaacccggagtacactaat	#1

SEQUENCE SIZE: 907
INCLUDED REGION SIZE: 907

PRODUCT SIZE: 399, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

```
    1 ccacacctaaaaaactttccacgtgaactgtcgttatttgttgtgtgtctgcgcgttgct

   61 ggcgtgcgtttgcttacgcttcggtgtttgcgagtgcgtgctggcggtgcgcggactgaa

  121 cgaaggtcgtgttgctgtgtgcctgctgcactgctgactttgcattgatttgcatggtct

  181 tggcggagcggcgggtgctgtgcgtgcgcggctgacttacttttttcaaaccccataccta

  241 acttactgattatactgtgagaacgaaagttcttgcttttaactagataacaactttcag

  301 cagtggatgtctaggctcgcacatcgatgaagaacgctgcgaactgcgatacgtaatgcg

  361 aattgcagaattcagtgagtcatcgaaattttgaacgcatattgcacttccgggttatgc

  421 ctggaagtatgtctgtatcagtgtccgtaaatcaaacttgcctttcttccttccgtgtag

  481 tcggtggaggagagttgcagatgtgaagtgtctcgctgtggttggtatatttgtttatgc
            >>>>>>>>>>>>>>>>>>>>

  541 acaacttgcgagtccttttaaatggacacgactttctctttttttgtatctgcgcggtgct

  601 gtgcgtgaacgcggtggttttcggatcgctcgcggctgtcggcgacttcggtgaatgcat

  661 tatggagtggacctcgattcgcggtatgttgggcttcggctggacaatgttgcttattgg

  721 gtgtctgttccgcgttcgccttgaggtgtactggtggctgtgggattgaactggttactg

  781 ttgttagtagtgggcgactcgttgtcgtgggtgcatctgtgtttttgtacttgtgcaatt

  841 ggcagaagaggagtctcaatttgggaaattagtgtactccgggtttgatcctgcgtgtat
                              <<<<<<<<<<<<<<<<<<<<
```

901 atctcaa

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS

		start	len	tm	gc%	any	3'	seq
1	LEFT PRIMER	490	21	55.99	47.62	4.00	2.00	gagagttgcagatgtgaagtg
	RIGHT PRIMER	889	21	56.26	42.86	4.00	1.00	atcaaacccggagtacactaa
	PRODUCT SIZE: 400, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00							
2	LEFT PRIMER	554	21	55.97	38.10	8.00	1.00	cctttaaatggacacgactt
	RIGHT PRIMER	888	21	56.26	42.86	4.00	2.00	tcaaacccggagtacactaat
	PRODUCT SIZE: 335, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00							
3	LEFT PRIMER	554	21	55.97	38.10	8.00	1.00	cctttaaatggacacgactt
	RIGHT PRIMER	889	21	56.26	42.86	4.00	1.00	atcaaacccggagtacactaa
	PRODUCT SIZE: 336, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 2.00							
4	LEFT PRIMER	363	21	55.90	38.10	6.00	3.00	ttgcagaattcagtgagtcat
	RIGHT PRIMER	726	21	56.25	38.10	3.00	2.00	agacacccaataagcaacatt
	PRODUCT SIZE: 364, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00							

#2

#3

Statistics

	con sidered	too many Ns	in tar get	in excl reg	bad GC%	no GC clamp	tm too low	tm too high	high any compl	high 3' compl	poly X	high end stab	
Left	5395	0	0	0	45	0	794	3259	0	8	38	54	1
Right	5344	0	0	0	1	0	625	3413	1	3	22	67	1

Pair Stats:
considered 463, unacceptable product size 442, high end compl 3, ok 18
primer3 release 1.1.0

(primer3_results.cgi 0.4.0 modified for WI)

Annex II